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WRR4, a broad spectrum TIR-NB-LRR gene from *Arabidopsis thaliana* that confers white rust resistance in transgenic oilseed brassica crops

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ABSTRACT

White blister rust caused by *Albugo candida* (Pers.) Kuntze is a common and often devastating disease of oilseed and vegetable brassica crops worldwide. Physiological races of the parasite have been described including races 2, 7 and 9 from *Brassica juncea*, *B. rapa* and *B. oleracea,* respectively; and race 4 from *Capsella bursa-pastoris* (the type host). A gene named *WRR4* was recently characterized from polygenic resistance in the wild brassica relative *Arabidopsis thaliana* (accession Columbia) that confers broad spectrum white rust resistance (*WRR*) to all four of the above *Albugo candida* races. This gene encodes a TIR-NB-LRR protein that, as with other known functional members in this subclass of intracellular receptor-like proteins, requires expression of the lipase-like defense regulator *EDS1*. Thus, we used RNAi-mediated suppression of EDS1 in a white rust resistant breeding line of *B. napus* (transformed with a construct designed from the *A. thaliana EDS1* gene) to determine whether defense signaling via EDS1 is functionally intact in this oilseed brassica. The eds1-suppressed lines were fully susceptible following inoculation with either race 2 or 7 isolates of *Albugo candida.* We then transformed white rust susceptible cultivars of *B. juncea* (susceptible to race 2) and *B. napus* (susceptible to race 7) with the *WRR4* gene from *A. thaliana*. The WRR4-tranformed lines were resistant to the corresponding *Albugo candida* race for each host species. The combined data indicates that *WRR4* could potentially provide a novel source of white rust resistance in oilseed and vegetable brassica crops.

INTRODUCTION

Arabidopsis thaliana has been an important genetic resource for investigating the molecular basis of genes that specify natural variation in disease resistance to bacterial, fungal, viral and oomycete pathogens of plants (Eulgem 2005, Ryan *et al.*, 2007, Holub *et al.,* 2001, 2008). As in most plant species, a majority of these so-called R-genes in *A. thaliana* encode intracellular receptor-like proteins that are characterized by a central nucleotide-binding (NB) domain and a C-terminal leucine-rich-repeat (LRR) domain. They can be further grouped into two subclasses based on either a TIR (similar to animal Toll-like/Interleukin-1 receptors) or coiled-coil (CC) domain at the N-terminus (Jones and Jones, 1997).

Most R-genes in *A. thaliana* confer disease resistance to a narrow spectrum of variants within a corresponding pathogen species and therefore may never be cost effective for commercial investment leading to the development of transgenic crops. However, important exceptions of broad spectrum disease resistance have been reported across a wide taxonomic range of plants including *A. thaliana* in which a single, dominantly expressed R-protein confers resistance to all known races of a pathogen. In an early report, a membrane bound receptor-like kinase designated Xa21 was described in rice that is effective against 29 diverse isolates of the bacterium *Xanthomonas oryzae* pv. *oryzae* (Wang *et al.*, 1996). Two genes in *A. thaliana* designated *RFO1* and *RPW8* encode other non-NB-LRR proteins that confer resistance against diverse collections of Fusarium or powdery mildew fungi, respectively (Diener and Ausubel 2005; Xiao *et al.*, 2001). In the *Solanaceae*, two broad spectrum CC-NB-LRR proteins have been discovered including Bs2 in pepper conferring black spot resistance to *X. campestris* pv *vesicatoria,* and RB (also named Rpi-blb1) from the wild species *Solanum bulbocastanum* that confers late blight resistance to current known races of the oomycete pathogen *Phytophthora infestans* in the US and in Europe (Song *et al.*, 2003; Van Der Vossen *et al.*, 2003). Similarly, TIR-NB-LRR genes have been reported including: *WRR4* from *A. thaliana* which confers resistance to four races of the oomycete *Albugo candida* (white blister rust) that occur naturally on other wild and domesticated host species including *Capsella bursa-pastoris* (the type host), *Brassica rapa, B. juncea* and *B. oleracea* (Borhan *et al.,* 2008); *RLM3* from *A. thaliana* which confers resistance to

several necrotrophic fungi (Staal *et al.*, 2008); and *RCT1* from *Medicago truncatula* which confers resistance to races of the anthracnose fungi *Colletotrichum trifolii* and *C. destructivum* (Yang *et al.,* 2008).

WRR4 provides an important example to test the transgenic use in crops of an R-gene that was originally derived from *A. thaliana*. *Albugo candida* (Pers.) Kuntze is an economically destructive crop pathogen. This biotrophic oomycete causes white blister rust in all vegetable and oilseed brassica crops such as *Brassica rapa* (Chinese cabbage and turnip rape; diploid A-genome), *B. oleracea* (cabbage, kale, broccoli and cauliflower; diploid C-genome), *B. juncea* (oilseed mustard; allotetraploid A and B genomes), and *Brassica napus* (oilseed rape; allotetraploid A and C genomes)(Fan *et al.,* 1983; Harper and Pittman 1974; Kumari *et al.,* 1970; Petrie 1988; Pound and Williams, 1963). The parasite emerges from infected tissue as white rust pustules to release asexual zoosporangia. These pustules can emerge on all aerial parts of the host, and are often associated with abnormal growth in surrounding tissue, stimulated by a hormonal imbalance, such as a common severe symptom called a "staghead" that occurs when the pathogen invades a floral stem. Susceptible oilseed crops (*B. napus* and *B. juncea*) are particularly vulnerable to floral infections with devastating losses (30-60% yield reduction) commonly occurring in North America, Australia, India and China (Bernier, 1972; Li *et al.,,* 1996, 2007; Petrie, 1973). Oilseed *B. juncea* has better drought tolerance and more durable stem canker resistance (*Leptosphaeria maculans*) than oilseed *B. napus* (Marcroft *et al.,* 2002), so canola-quality *B. juncea* varieties are being developed to extend production in low-rainfall areas of Australia and North America (Li *et al.,* 2007). Unfortunately, white rust looms as a major threat to *B. juncea* production in these areas.

Physiological races of *Albugo candida* have been described which each have narrow host ranges including: races 2, 7 and 9 from *B. juncea, B. rapa* and *B. oleracea*, respectively *(*Pound and Williams, 1963; Hill *et al.,* 1988). The type specimen of *Albugo candida* was collected from the invasive species *Capsella-bursa pastoris*, and was later designated as race 4. These races are highly specialized, however, they are not necessarily restricted to the host species from which they were originally collected. For example, isolates of race 7 collected from *B. rapa* can cause disease in many cultivars of *B. napus* under field conditions (Bernier, 1972; Fan *et al.,* 1983). Similarly, a low

frequency (less than 10%) of *A. thaliana* accessions are susceptible to standard isolates of *Albugo candida* races 2, 4 and 7 in a conducive laboratory environment (Borhan *et al.,* 2008; Holub, unpublished). In contrast, *A. thaliana* is universally a non-host of *Albugo candida* race 9. It is important to note here that a molecular taxonomic distinction has been made between *Albugo candida* and a species now called *Albugo laibachii* that commonly causes white rust in *A. thaliana* under natural field conditions (Holub *et al.,* 1995; Rehmany *et al.,* 2000; Voglmayr and Riethmüller, 2006; Thines *et al.,* 2009). We have previously referred to this common parasite of wild *A. thaliana* as *Albugo candida* subsp. *arabidopsis* (Borhan *et al*., 2008).

WRR4 was identified as a gene in *A. thaliana* Columbia (Col) which confers full immunity to *Albugo candida* races 2, 4 and 7 when stably transformed into the accession Wassilewskija (Ws); this accession is susceptible in the laboratory to all three races (Borhan *et al.*, 2008). *WRR4* also improved the partial resistance of Ws to race 9, conferring full immunity in transgenic lines. RNAi suppression of the defense regulator protein EDS1 conferred full susceptibility to race 2, indicating that WRR4 is fully dependent on expression of this lipase-like protein (Borhan *et al*., 2008; Parker *et al.,* 1996). However, the same eds1-suppression lines exhibited enhanced colonization to varying degrees by races 4, 7 and 9 but still exhibited residual resistance that restricted the formation of rust pustules (least restricted with race 7, most restricted with race 9). This indicates that Col contains additional WRR genes which are EDS1-independent.

The purpose of the research described below was to determine whether *WRR4* from *A. thaliana* can confer white rust resistance in a brassica species. We began transgenic experiments using RNAi-mediated suppression of EDS1 in a white rust resistant breeding line of *B. napus* (transformed with a construct designed from the *A. thaliana EDS1* gene) to confirm beforehand whether defense signaling via EDS1 is functionally intact in this crop species. We then transformed white rust susceptible cultivars of *B. napus* (susceptible to race 7) and *B. juncea* (susceptible to race 2) with the *WRR4* gene from *A. thaliana*.

RESULTS

Resistance to *Albugo candida* races 2 and 7 in *B. napus* is EDS1 dependent

We searched a *B. napus* EST database containing nearly 150,000 ESTs (generated at SRC, AAFC) for sequences that shared homology with *A. thaliana* EDS1 (referred to hence as At.EDS1). Eight *B.napus* ESTs were identified, and sequence assembly resulted in a single full length ORF of 1800 bp that encodes a lipase-like protein (referred to hence as Bn.EDS1). The DNA sequence of At.EDS1 and Bn.EDS1 share 71% identity when aligned, whereas the protein sequences share 61% identity (Figures 1& 2).

Given the close homology between these two genes, we used the At.EDS1-RNAi construct described by Borhan *et al*., (2008) to suppress EDS1 in a white rust resistant, doubled haploid breeding line of *B. napus* (DH12075). Three independent RNAi transformed lines were identified using the herbicide selection marker phosphinotricin and confirmed by PCR. Seven-day-old seedlings of each T_1 line were inoculated with *Albugo candida* isolate Ac7a, and each family segregated in approximately a 3 to 1 ratio of resistant (no pustules) and susceptible phenotypes (large, profuse pustules), respectively (Table 1; Figure 3). We then tested T2 lines of susceptible T1 generation from each independent transformant. These lines were uniformly susceptible to *Albugo candida* isolate Ac2v (Table 1). The combined data from RNAi suppression lines indicates that the natural white rust resistance in DH12075 to *Albugo candida* races 2 ands 7 is dependent on EDS1 expression.

WRR4 from _A. thaliana_ confers full resistance in transgenic _B. napus_ and _B. juncea_

To test whether the *WRR4* gene from *A. thaliana* can confer white rust resistance in *B. napus* and *B. juncea,* we transformed a susceptible accession of each oilseed crop including the *B. juncea* cultivar Cutlass which is susceptible to Ac2v, and the *B. napus* breeding line ACS-N32 which is susceptible to Ac7a.

Seven independent transgenic lines of *B. juncea* cv Cutlass were identified containing a single insertion of *WRR4* from *A. thaliana* using the selectable herbicide marker phosphinotricin. We confirmed the number of insertions by PCR and Southern blots (Table 2). T_2 seeds were harvested from each transgenic line. Seven-day-old T_2 seedlings were inoculated with Ac2v, and some segregated in ca. 3:1 ratio for resistance to susceptible (Table 2), which confirmed single insertion of *WRR4* in each line. Growth of the parasite in a resistant transformant was usually localized to 1-3 penetrated cells at

the site of infection (Figure 4). However, a necrotic patch of cells was occasionally visible macroscopically, which under the microscope, was associated with more extensive growth of hyphae. A minute pustule rarely developed from these confined, necrosis-inducing colonies of *Albugo candida.* The necrotic patch phenotype may be indicative of a heterozygous genotype, however, this was not confirmed.

Similarly, three transgenic lines from *B. napus* line ACS-N32 were obtained containing the WRR4 gene from *A. thaliana.* T_2 progeny from these lines were inoculated with *Albugo candida* isolate Ac7a, and segregated for resistance (Table 2). As in *B. juncea,* the WRR4-mediated resistance was typically associated with restriction of parasite at the site of infection (Figure 5).

DISCUSSION

Two decades of *A. thaliana* molecular biology has been central in shaping our current understanding of innate defense in plants and has also had an impact on understanding infectious disease in animals (Chisholm *et al.,* 2006; Dangl and Jones, 2001; Holub, 2007; Jones *et al.,* 2008). Early contributions to crop improvement came from a precedent that a virulent bacterial pathogen could be genetically modified to deliver an avirulence effector from a crop pathogen and then used as a physiological probe to identify the matching receptor-like R-gene from a non-host of the crop pathogen (Gassmann *et al.,* 1999; Holub, 2001; Warren *et al.,* 1998). This method holds great promise for transient delivery of avirulence effectors from filamentous pathogens (Rentel *et al.,* 2008; Sohn *et al.*, 2007; Vleeshouwers *et al.,* 2008). More importantly, the use of molecular markers derived from conserved domains in the most common NB-LRR R-genes and other essential defense response genes is enabling marker-assisted selection of homologs in crops (Aarts *et al.,* 1998; Botella *et al.,* 1997; Leister *et al.,* 1996; McHale *et al.*, 2009; Shen *et al.,* 1998). Lettuce provides a superb illustration of how even minor crops will benefit from this combined "model-to-crop" knowledge transfer (Caldwell and Michelmore, 2009; McHale *et al.*, 2009; Wroblewski *et al.,* 2009).

Surprisingly, the direct use of *A. thaliana* R-genes in transgenic crops has not been demonstrated, despite several examples of genes that could potentially confer broad spectrum resistance to brassica crop pathogens (Cooley *et al.,* 2000; Grant *et al.,* 1995;

Staal *et al.,* 2006, 2008; Xiao *et al.,* 2001). Thus, the report here that *WRR4* from *A. thaliana* confers white rust resistance in two oilseed brassica crops provides another important precedent for the utility of *A. thaliana* research. *Albugo candida* can cause severe yield loss in oilseed and vegetable crops of *B. juncea, B. oleracea* and *B. rapa,* for example with losses in oilseed mustard (*B. juncea*) often reaching 60% for small-holding farmers in India (Bernier, 1972; Li *et al.,* 2007; Li *et al.,* 1996; Petrie, 1973). *B. juncea* has better drought tolerance and more durable stem canker resistance (*Leptosphaeria maculans*) than *B. napus* oilseed rape (Marcroft *et al.,* 2002), and canola-quality *B. juncea* varieties have therefore been developed to extend oilseed production in low-rainfall areas of Australia and Canada (Li *et al.,* 2007). Unfortunately, white rust looms as a major threat to production in these areas.

The durability of a single broad spectrum R-gene for disease control in crops is not guaranteed but will instead depend on how readily the pathogen can evolve variants to overcome the host resistance (Leach *et al.,* 2001). Pathogen effector molecules have been identified that correspond as the avirulence effector detected by broad spectrum R-proteins including Bs2 from pepper, and Rb from potato (Kearney and Staskawicz, 1990; Vleeshouwers *et al*., 2008). The corresponding avirulence effectors in these examples occur species-wide in each respective pathogen and appear to be significantly constrained from evolving due to a high penalty of mutation. Thus, slow or non-evolving effectors is a plausible expectation that warrants investigation in pathogens from the other examples of broad spectrum R-proteins, such as the predicted avirulence effector corresponding with *WRR4* that may be shared amongst physiological races of *Albugo candida* (Borhan *et al.,* 2008).

NB-LRR *R*-genes may have evolved and expanded so extensively as a gene family in plants because they typically induce defense only when its likely to be beneficial after detection of an avirulent pathogen, and they are unlikely to confer susceptibility to non-target microorganisms. *R*-genes such as *Bs2, Rb, RLM3* and *WRR4* provide the additional advantage of broad spectrum disease control, and illustrate the potential for expanding the use of NB-LRR genes from wild species in crop improvement. Interestingly, a trade-off in enhanced susceptibility to non-target

pathogens has been observed for broad spectrum resistance genes that do not encode NB-LRR proteins (Jarosch *et al*., 2003; Wang *et al.,* 2007).

Genetic improvement of multiple agronomic traits (*e.g.,* drought tolerance, nutrient-use efficiency, yield performance and disease resistance) in crops that have large and complex genomes will continue to benefit from under-pinning investment in model plants (Bevan and Waugh, 2007). *A. thaliana* is in particular an excellent tool for crop scientists working with brassica crops considering the significant synteny between the two genomes (Schranz *et al*., 2007). The results from transgenic suppression of EDS1 and gain-of-function in resistance to *Albugo candida* with *WRR4* indicate that brassica species contain the genes essential for the direct use of NB-LRR encoding other R-genes from *A. thaliana* in brassica crops such as the broad spectrum stem canker resistance genes *RLM1* and *RLM3* (Staal *et al.,* 2006, 2008). The advantage in both the white rust and stem canker examples is that economically devastating crop pathogens were strategically used from inception in the molecular genetics research of *A. thaliana*.

MATERIALS AND METHODS

Pathogen handling and inoculation

Albugo candida races were propagated on the appropriate susceptible host (Rimmer *et al*., 2000). Pustules were harvested at 10-14 dai and stored at -20 °C or used fresh to prepare the inoculum by suspending the spores in dH_2O at a concentration of $2x10^4$/ml sporangia. Inoculum was incubated at 14-16 °C for 2-4hrs to ensure the release of motile zoospores. This inoculum was kept on ice during inoculation of 5-7 day-old brassica seedlings. A repeater pipette was used to place a 10ul drop of the inoculum on each half of a cotyledon. Inoculated plants were kept at 14-16 °C under a micro-propagator, to maintain humidity. After 24hrs plants were transferred to a growth chamber with 12 hrs light, 18 °C temperature during the night and 20 °C daytime. Cotyledon responses to *Albugo candida* were scored at 7 to 14 dai.

Microscopy of infected tissues

To prepare inoculated tissues for microscopy observation of pathogen growth and plant response, cotyledons were excised from the seedlings at 7-14 dai, placed in a 50 ml Falcon tube submerged in trypan blue (Parker *et al.*, 1996) and transferred to a boiling water bath for 20 min. After this, stain was replaced with chloral hydrate. Tissues were left in chloral hydrate for 24 hrs cleared tissues were placed on a glass slide in 50% glycerol, covered with a coverslip and observed using Differential Interference Contrast (DIC) with a Zeiss Axio Imager Z1 microscope.

Brassica Transformation.

Hypocotyl explants of *B. napus* or *B. juncea* were used for *Agrobacterium tumefaciens* (GV3101pMP90)-mediated transformation (De Block *et al*., 1989). Plants were grown in the tissue culture growth room conditions (22 ± 1°C under 16 h light, 100 µE/m2/sB). Transgenic plants were selected based on resistance to the herbicide phosphinotricin, transferred to soil and were grown in the greenhouse (16 h light/8 h dark, 20°C/17°C). Transgenic plants were further confirmed by PCR. WRR4 construct used for transformation have been previously described by Borhan *et al*., (2008).

Generating B. *napus* EDS1-RNAi lines

The EDS1-RNAi construct described by Borhan *et al*., (2008) was transferred to the *B. napus* double haploid line DH12075 which is naturally resistance to *Albugo candida* races 2 and 7. Response of seedlings of EDS1 suppressed lines was monitored at 7- 14 dai with Ac2 or Ac7.

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Table 1. RNAi suppression of EDS1 and white rust resistance in *Brassica napus*. EDS1 is a lipase-like protein that is commonly required for disease resistance conferred by TIR-NB-LRR *R*-genes in *Arabidopsis thaliana*. Suppression of EDS1 was achieved using a construct designed from the *A. thaliana EDS1* gene. Transgenic lines were tested for susceptibility to *Albugo candida* races 2 (isolate Ac2v) and 7 (Ac7a) as follows: T_1 seedlings of three independent lines were assessed for resistance (R) or susceptibility (S) to Ac7a at nine days after inoculation (dai); and T_2 seedlings derived from homozygous Ac7a-susceptible T_1 lines were assessed for response to Ac2v at 9 dai.

Host Generation	Albugo Isolate	Line No.	Total	R	S	χ^2	P
T_1	Ac7a	211	30	19	11	2.17	0.14
		415	37	27	10	0.08	0.77
		416	40	32	8	0.53	0.46
T_2	Ac2v	211-305	37	0	37		
		416-240	47	0	47		

Table 2. Segregation of white rust resistance amongst progeny of *Brassica juncea* cv Cutlass and *B. napus* line ACS-N32 following transformation with the *WRR4* TIR-NB-LRR gene from *Arabidopsis thaliana*. The number of transgene insertions in the T_1 generation was determined by southern blotting. The number of resistant (R; green cotyledon and no rust pustules) and susceptible (S; large pustules formed profusely on underside of cotyledons) T_2 seedlings were recorded at 10 dai.

Brassica species	Line No.	No. of T_1 insertions	Total T_2	R	S	χ^2	P
B. juncea	1	1	42	30	12	0.28	0.59
	2	2	41	32	9	0.20	0.65
	3	2	35	23	12	1.60	0.20
	4	1	41	37	4	5.08	0.02
	5	4	9	9	0	3.00	0.08
	6	1	40	27	13	1.20	0.27
	7	1	40	31	9	0.13	0.71
	8	1	41	27	14	1.82	0.17
B. napus	1	4	41	37	4	5.08	0.02
	2	1	39	22	17	7.18	0.007
	3	1	25	18	7	0.12	0.72

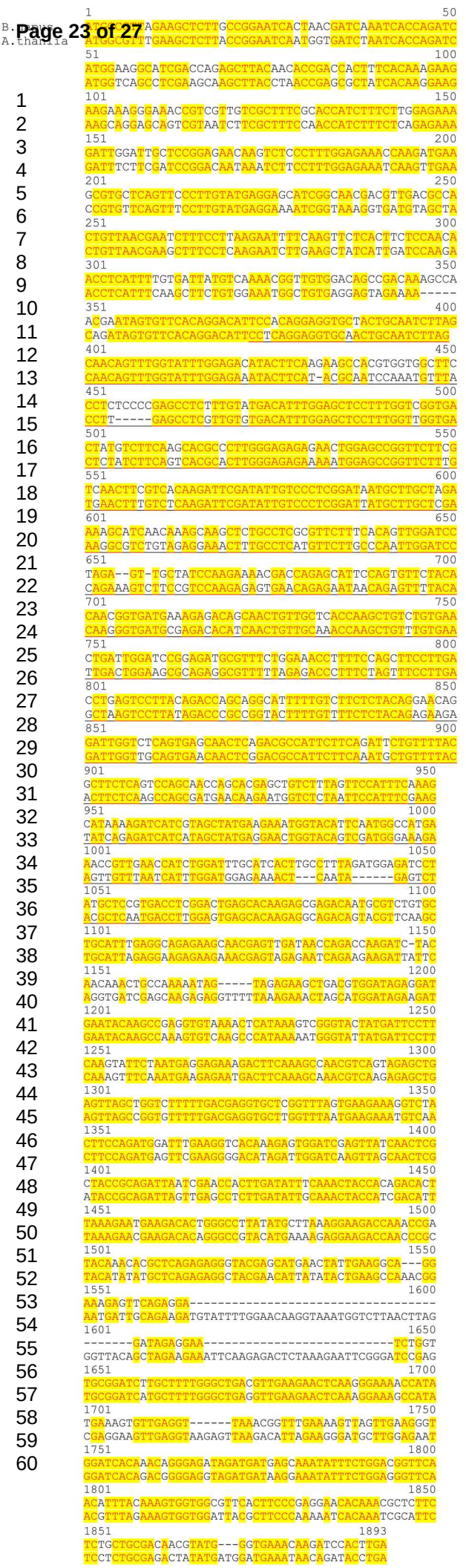
Figure 1. Alignment of *Brassica napus* EDS1 homologue and *Arabidopsis thaliana EDS1* ORFs. Blastn search of *B. napus* sequence database identified eight ESTs with homology to the Arabidopsis *EDS1*. Assembly of these ESTs resulted in a cDNA of 2043 bp and an ORF of 1803 bp encoding a lipase-like protein. Underlined sequences shows the interval of Arabidopsis *EDS1* used for suppression of Brassica *EDS1* by RNAi.

Figure 2. Alignment of *Brassica napus* protein with homology to the lipase-like protein EDS1 in *Arabidopsis thaliana* which in required for several examples of TIR-NB-LRR-mediated disease resistance. The *B. napus* EDS1 open reading frame encodes a 600 aa protein of 68.6 KDa with 70% similarity and 61% identity to *A. thaliana* EDS1.

Figure 3. White rust resistance to two avirulent isolates of *Albugo candida* (Ac2V and Ac7A) is suppressed in *Brassica napus* line DH12075 with RNAi knock-out expression of the lipase-like defense gene *EDS1*. One-week-old seedlings were infected with either isolate and interaction phenotypes (for Ac7A, shown here) were photographed ten days later. **A, B** top and bottom, respectively of a fully resistant wild-type cotyledon. **C, D** top and bottom of a cotyledon from a fully susceptible EDS1-RNAi suppressed line.

Figure 4: *Arabidopsis thaliana WRR4,* confers full resistance in the susceptible *B. juncea* cv cutlass. Picture cotyledons inoculated at with the virulent isolate Ac2v at 10 days after inoculation. **A**, Wild type cutlass exhibiting full susceptibility with profuse development of white pustules on the lower surface and no host cell necrosis visible microscopically (far right). **B** to **D** cotyledons of *WRR4* transgenic cutlass showing resistance phenotypes. **B**, indicating full resistance with no formation of pustules on upper or lower surfaces of the cotyledon minute patches of necrotic cells visible microscopically at the site of infection (far right). **C**, necrotic patches with no formation of pustules on upper or lower surfaces of the cotyledon and restriction of hyphae to cells surrounding the site of infection as shown by microscopy (far right). **D**, necrotic patch with few minute sporadic pustules and restriction of hyphae to cells surrounding the site of infection as shown by microscopy (far right).

Figure 5. The white rust resistance gene *WRR4* from *Arabidopsis thaliana* confers full resistance in a susceptible cultivar of *Brassica napus* (ACS-N32). **A**, upper and lower surfaces of a wild type cotyledon 10 days after inoculation with the virulent isolate Ac7a, exhibiting full susceptibility with profuse development of white pustules on the lower surface and no host cell necrosis visible microscopically (far right). **B,** upper and lower surfaces of an ACS-N32 cotyledon stably transformed with *WRR4*, indicating full resistance with no formation of pustules on upper or lower surfaces of the cotyledon and minute patches of necrotic cells visible microscopically at the site of infection (far right).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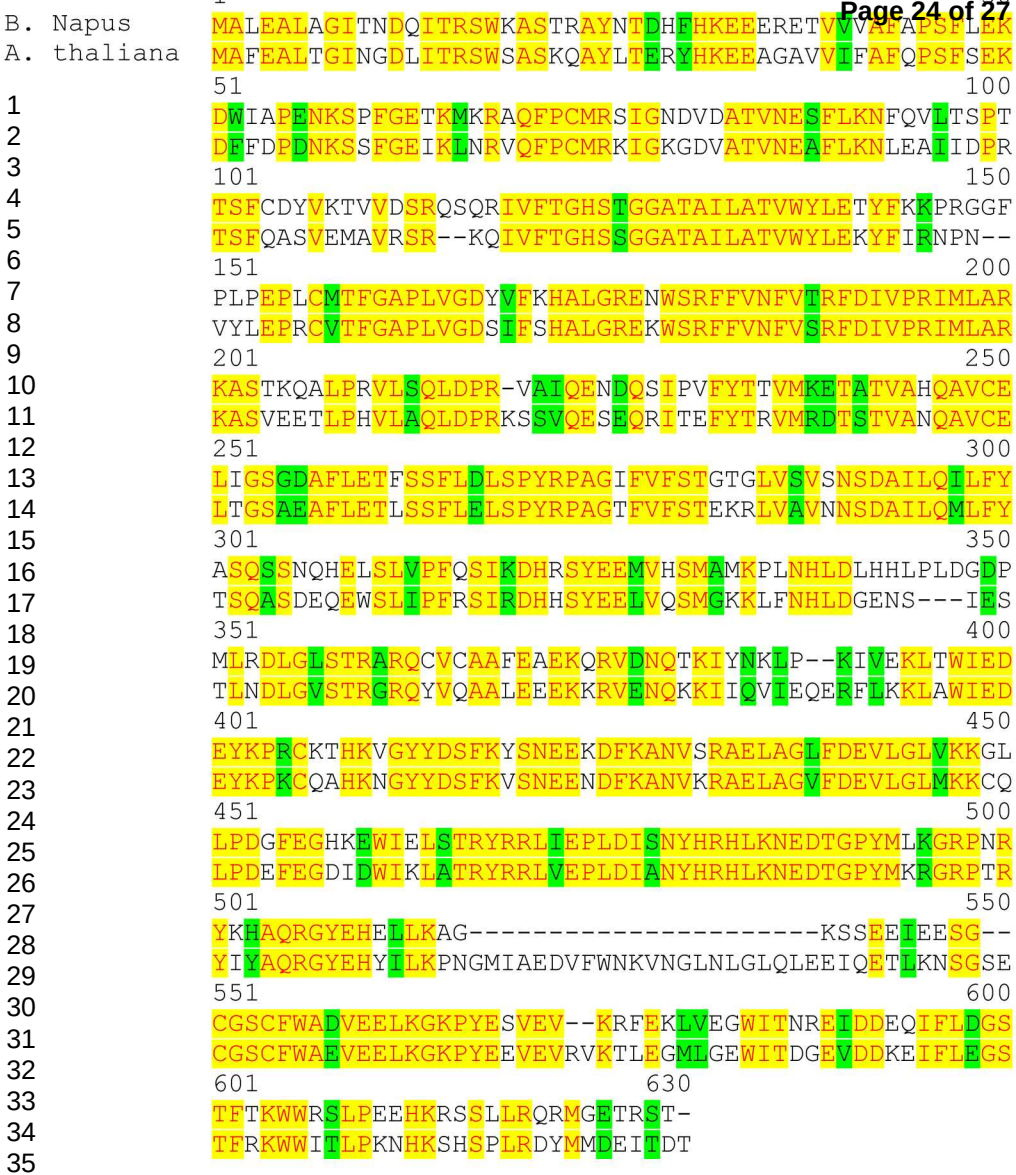
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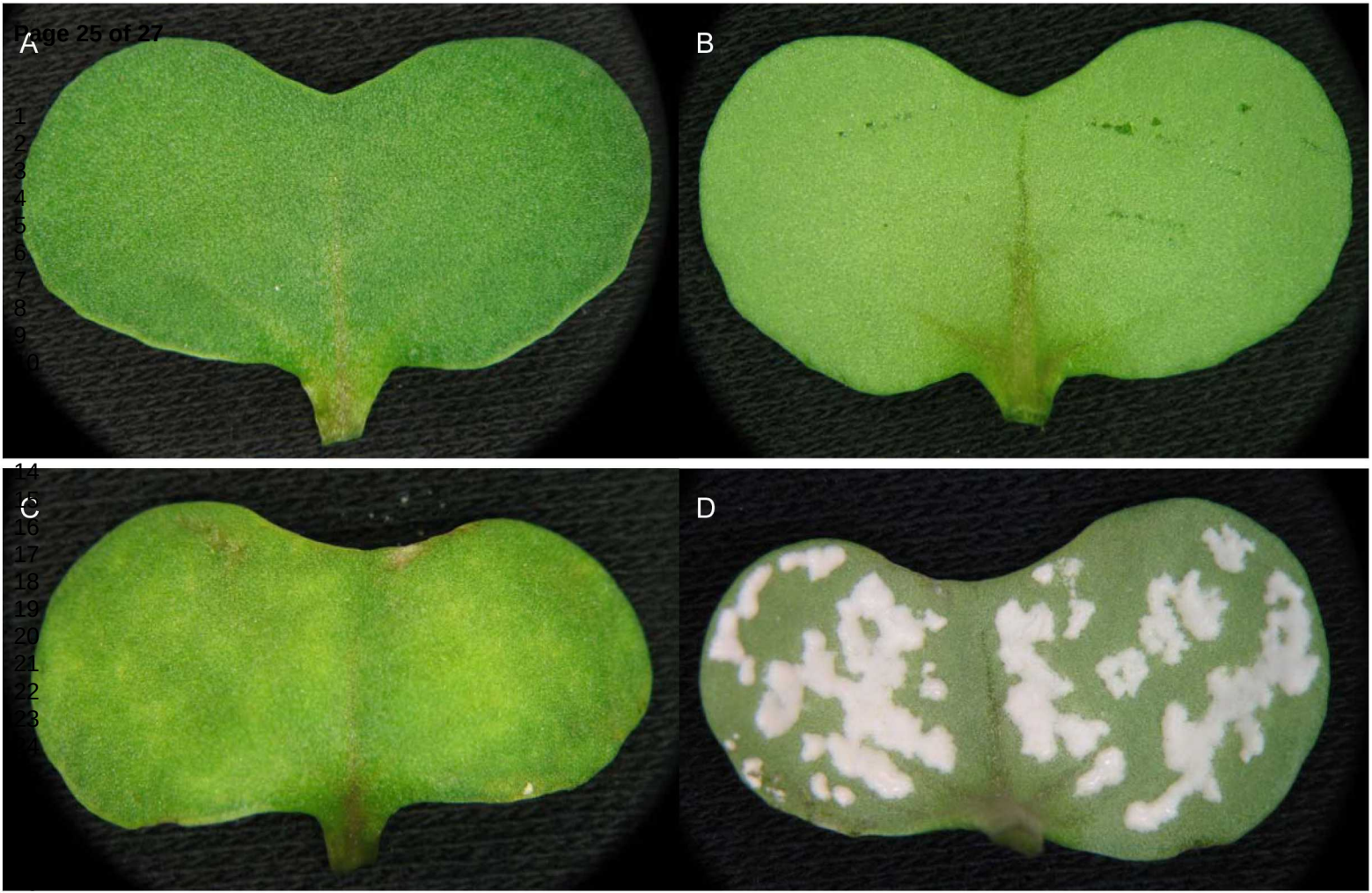
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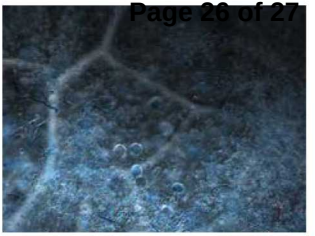
A

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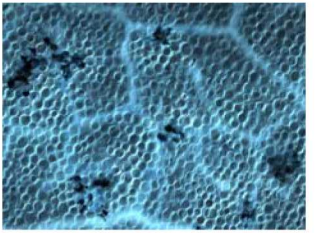


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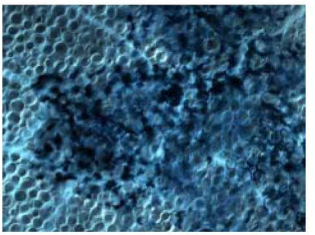


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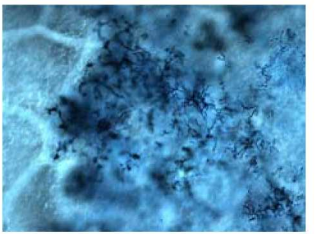

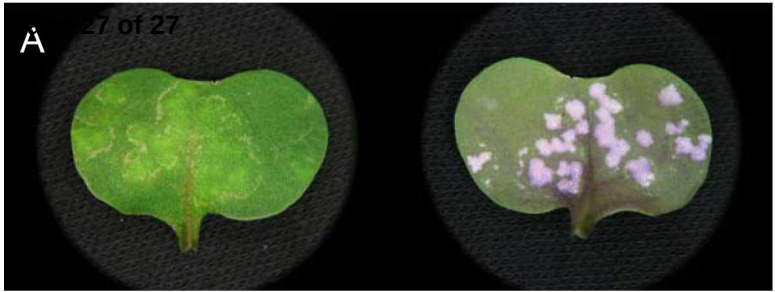

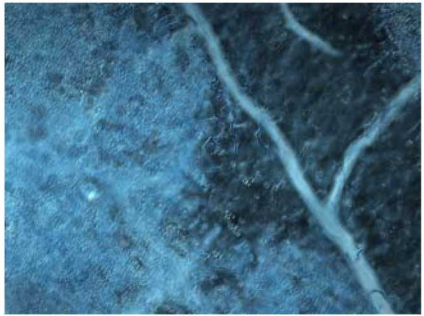

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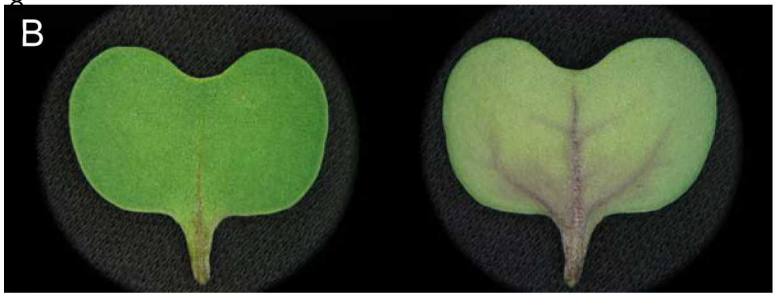

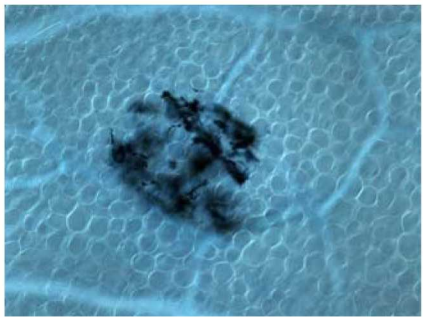

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